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Exposure of Adult Female Mice to Low Doses of di(2-ethylhexyl) Phthalate Alone or in an Environmental Phthalate Mixture: Evaluation of Reproductive Behavior and Underlying Neural Mechanisms

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Background: We have previously shown that adult male mice exposure to low doses of an ubiquitous endocrine disruptor, di(2-ethylhexyl) phthalate (DEHP), alters courtship behavior.

Objective: The effects of adult exposure to low doses of DEHP alone or in an environmental phthalate mixture on estrous cyclicity, reproductive behavior, and underlying neural structures were analyzed in female mice.

Methods: Two-month-old C57BL/6J females were exposed orally for 6 wk to DEHP alone (0, 5 or 50 μg/kg/d) or to DEHP (5 μg/kg/d) in a phthalate mixture. Estrous cyclicity was analyzed in intact mice, and behavior [lordosis, olfactory preference, partner preference, ability to stimulate male ultrasonic vocalizations (USVs)] was measured in ovariectomized mice primed with estradiol and progesterone. Immunohistochemical studies were conducted in the neural structures involved in behavior for estrogen receptor (ER) α and progesterone receptor (PR).

Results: Exposure to DEHP alone or in mixture lengthened the estrous cycle duration, with a shorter proestrus and longer estrus and metestrus stages. Under normalized hormonal levels, females exposed to DEHP alone or in mixture exhibited altered olfactory preference. A lower lordosis behavior and ability to attract and stimulate male emission of courtship USVs was observed, probably due to modifications of pheromonal emission in exposed females. The behavioral alterations were associated with a lower number of PR-expressing neurons, without changes in ERα, in the neural circuitry underlying sexual behavior. The majority of effects observed was comparable between the two DEHP doses and were driven by DEHP in the mixture.

Conclusions: Exposure to environmental doses of DEHP alone or in mixture altered several components of female sexual behavior in mice, probably through selective disruption of neural PR signaling. Together with the previously reported vulnerability of male mice, this finding suggests a major impact of exposure to phthalates on sexual reproduction, including in other species with similar neural regulatory processes. https://doi.org/10.1289/EHP7662

Introduction

Phthalates are among the most frequently detected organic pollutants in the environment (Gao and Wen 2016), due to their extensive use as plasticizers in several commonly used products. Di(2-ethylhexyl) phthalate (DEHP) is the most abundant molecule of this family (Gao and Wen 2016). Previous studies in humans reported associations between phthalate metabolites in urine and reduced anogenital distance in boys (Bornehag et al. 2015) and interest in sexual activity in women (Barrett et al. 2014) or altered age of pubertal onset in girls (Berger et al. 2018). Experimental studies using rodents described adverse effects of developmental exposure to DEHP on sexual differentiation of the urogenital tract in males, age of puberty and testicular and ovarian functions (for review: Hannon and Flaws 2015; Howdeshell et al. 2008; Rowdhwal and Chen 2018). However, the potential effects of adult exposure to DEHP on the neural regulation of reproductive behavior have received less attention. In this context, we previously showed that exposure of adult male mice to DEHP at the tolerable daily intake dose (TDI) of 50 μg/kg/d (EFSA 2005, 2019) or at lower doses close to the environmental exposure altered courtship behavior (Dombret et al. 2017). In particular, DEHP exposure lowered the emission of ultrasonic vocalizations (USVs) and the ability to attract females and delayed the initiation of mating. In female rodents, previous studies reported that acute exposure to DEHP during adulthood alters estrous cyclicity and ovarian function (Chiang et al. 2020; Davis et al. 1994; Hannon et al. 2014; Herreros et al. 2013; Li et al. 2012). Whether and how adult exposure to low environmental doses of DEHP affects female behavior and underlying neural structures remains to be investigated. Indeed, the effects of exposure to phthalates on female sexual behavior were analyzed only for perinatal exposure (Guerra et al. 2010; Lee et al. 2006).

In female rodents, the expression of sexual behavior is limited to the estrus phase of the cycle, coinciding with ovulation (for review: Mhaouty-Kodja et al. 2018). This behavior is induced by a hormonal sequence involving the preovulatory surge of estradiol, which triggers both the ovulatory surge of pituitary luteinizing hormone (LH) and, via an estrogen receptor (ER) α-mediated action, the upregulation of progesterone receptor (PR). Progesterone liberated following ovarian stimulation by LH induces female receptivity. Female sexual behavior includes an attractivity phase during which the female stimulates male behavior by emitting pheromones and a copulatory phase with the female adopting a receptive posture called lordosis when being approached from behind for insemination by the courting male. All these behavioral patterns are controlled by a neural circuitry involving the olfactory bulb, which transmits chemo-signals to the medial and posteromedial cortical amygdala and then to the bed nucleus of the stria terminalis and the ventromedial hypothalamus. This principal facilitatory system for lordosis behavior is activated by estradiol and progesterone during the estrus phase, as mentioned above. Inversely, the constraints exerted by the inhibitory system involving the hypothalamic preoptic and arcuate nuclei are lifted during this period.

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In the present study, we characterized the effects of chronic exposure of adult female mice to DEHP alone or in an environmental phthalate mixture on reproductive behavior and underlying neural structures. For this purpose, adult C57BL/6J female mice were assigned to one of four exposure groups. The first three groups included females exposed orally for 6 wk to the vehicle (control), DEHP at the TDI dose of 50 μg/kg/d, or DEHP at 5 μg/kg/d. The DEHP dose of 5 μg/kg/d is within the environmental exposure range; this dose induced behavioral alterations in male mice following adult or pubertal exposure (Capela and Mhaouty-Kodja 2021; Dombret et al. 2017). To mimic the environmental coexposure to phthalates (Martine et al. 2013; Anses 2015), the fourth group of females was exposed to a phthalate mixture containing DEHP at 5 μg/kg/d, dibutyl phthalate (DBP) at 0.5 μg/kg/d, butylbenzyl phthalate (BBP) at 0.5 μg/kg/d, diisobutyl phthalate (DiBP) at 0.5 μg/kg/d and diethyl phthalate (DEP) at 0.25 μg/kg/d. A first cohort of female mice including the four treated groups was analyzed for estrous cyclicity and body and uterine weights. A second cohort of females was ovariectomized and primed with estradiol and progesterone to induce their receptivity (acceptance of male mounting and display of lordosis behavior in response to mounts) under similar hormonal conditions. These females were analyzed for their lordosis and rejection behaviors as well as for olfactory preference in the presence of sexually experienced males. The ability of females and their pheromonal cues to attract male partners and induce the male emission of courtship USVs was also investigated. In this second cohort, locomotor activity was measured, and body weight (BW) was monitored during the whole period of treatment and behavioral analyses. The neural structures involved in the expression of sexual behavior and belonging to the facilitatory and inhibitory systems of lordosis behavior were studied for the number of ERα- and PR-immunoreactive neurons and mean fluorescence density.

Methods

Animals and Treatments

Studies were performed in accordance with the French and European legal requirements (Decree 2010/63/UE) and were approved by the "Charles Darwin" Ethical committee (project number 01490-01). The experiments were reported following the Animal Research: Reporting *In Vivo* Experiments (ARRIVE) guidelines.

Animals were obtained from breeding of male and female C57BL/6J mice (Janvier Labs) and housed in nest-enriched polysulfone cages with polysulfone bottles. Mice were kept at 22°C under an inverted light schedule, i.e., the dark time began at 1330 hours (1:30 P.M.) with a 12:12 h light–dark cycle, and fed a standard diet (A03–10; Safe-diets) with free access to food and water. Offspring were mixed at weaning to avoid potential litter effects (no more than one female per litter per cage, and one to two females per litter in each treatment group) and allowed to grow until 8 wk of age.

Oral exposure was performed as previously described (Dombret et al. 2017). Phthalates (Sigma-Aldrich) were dissolved in ethanol and water (1% and 40% of prepared food, respectively) and incorporated by the experimenter into powdered food (A03–10; Safe-diets) that was then reconstituted into pellets. Eight-week-old females were fed *ad libitum* with chow containing the vehicle [i.e., ethanol and water (1% and 40% of prepared food, respectively; control group)], DEHP (CAS 117-81-7) at 50 or 5 μg/kg/d (DEHP-50 and DEHP-5 groups, respectively), or a phthalate mixture (Mix group) containing DEHP at 5 μg/kg/d, DBP (CAS 84-74-2) at 0.5 μg/kg/d, BBP (CAS 85-68-7) at 0.5 μg/kg/d, DiBP (CAS 84-69-5) at 0.5 μg/kg/d, and DEP (CAS 84-66-2) at 0.25 μg/kg/d. The composition of the phthalate mixture was based on French and European studies showing an external coexposure to these molecules (Martine et al. 2013) and the presence of their metabolites in urinary samples (Anses 2015; Dewalque et al. 2014). The ratio of DEHP over the other phthalates was determined on the basis of the estimated daily intake in France and Europe (Dewalque et al. 2014; Martine et al. 2013). Mice were weighed weekly, and phthalate doses were adjusted to their BWs and calculated for a daily food intake of 5 g per animal. Reconstituted pellets were prepared every week immediately after animal weighing, stored at 4°C, and changed twice a week. Analyses started after 6 wk of exposure, and treatments were maintained during the whole period of the study.

Experiments were conducted on two cohorts of female mice, each including 4 treated groups. The first cohort including 15 intact females per treatment group was subjected to analyses of estrous cyclicity; body and uterine weights were collected from all these females at necropsy (Figure S1). Behavioral analyses were conducted on a second cohort including 11 females from the control group, 12 from the DEHP-5 group, 13 from the DEHP-50 group, and 13 females from the Mix group, which were all ovariectomized and hormonally primed (Figure S1). At the end of behavioral analyses, females were sacrificed, and body and uterine weights were measured; the brains collected from 6 females per treatment group were processed for immunohistochemical analyses. All analyses were performed by blind observation, because females were identified by numbers attributed at weaning without any information concerning their treatment details.

Estrous Cyclicity

Six weeks after the beginning of exposure, analyses of the estrous cycle were started while maintaining the treatment. Vaginal smears flushed with physiological saline were taken daily from females for 7 wk. The estrous cycle phases were identified by microscopy after hematoxylin/eosin coloration of the vaginal smears. The cycle duration was calculated as the average mean of days spent in seven complete cycles. The number of days spent in each stage of the estrous cycle was also determined. BW was examined during the whole period of treatment and estrous cycle analyses. The estrous cycle was monitored until sacrifice by pentobarbital injection (120 mg/kg) to measure body and uterine weights of females at the metestrus stage.

Behavioral Tests

Four weeks after the beginning of exposure, female mice were ovariectomized under general anesthesia (xylazine 10 mg/kg and ketamine 100 mg/kg). At the time of ovariectomy, all females received 1 cm subcutaneous SILASTIC™ implants (3.18 mm outer diameter × 1.98 mm inner diameter; Dow Corning) filled with 50 μg of estradiol benzoate (Sigma-Aldrich) in 30 μL sesame oil and sealed at each end with SILASTIC™ adhesive as previously described (Dombret et al. 2017; Naulé et al. 2014, 2015; Raskin et al. 2012). Two weeks later, behavioral analyses were started, and each female was given a subcutaneous injection of 1 mg/100 μL progesterone (Sigma-Aldrich) dissolved in sesame oil 4–5 h before each test to induce female receptivity. Tests were conducted under red-light illumination, 2 h after lights off and were videotaped for later analyses. They were conducted following the order indicated below and in Figure S1, starting with lordosis, then olfactory preference, partner preference, USV analysis, and ending with locomotor activity. All females of the second cohort were analyzed in the behavioral tests. Control untreated males were sexually experienced before the beginning

of tests, which were performed with a different male per female (lordosis and USV tests) or per a pair of females or urine (partner preference tests). The devices used in the tests, with the exception of animal home cages, were cleaned with 10% ethanol between trials.

Lordosis. Females were tested twice: in a first test (naive) and 2 wk later after this first sexual experience in a second test (sexually experienced). Each female was introduced into the cage of a sexually experienced male used as a partner. Tests ended after 20 min. The percentage of females exhibiting lordosis behavior, lordosis quotient (lordosis number/number of mounts) and rejection quotient (rejection number/number of mounts) were calculated for each subject in response to male mounting (Naulé et al. 2014, 2015).

Olfactory preference. Olfactory preference was assessed in an enclosed plexiglass Y-maze as previously described (Capela et al. 2018; Dombret et al. 2017; Picot et al. 2014). Female mice were allowed to become familiar with the maze, where two empty perforated goal boxes were placed at each end, for 10 min over two consecutive days. On the day of the test, females were offered the choice between a sexually receptive female and a gonadally intact male, which were placed in the goal boxes. Stimuli were anesthetized to avoid any social interaction. Exposed females did not have direct access to these stimuli, but the perforated walls of goal boxes allowed air to flow from the boxes into the maze. The total time spent in chemo-investigation and the number of entries into each arm of the maze were scored during the 10-min test. The discrimination index was calculated as the time spent by exposed females in male investigation (M) minus the time spent in female investigation (F) divided by the total time of investigation (M-F)/(M+F).

Partner Preference Tests

Three-chamber test. Sexually experienced males were allowed to become familiar, for 10 min over 2 consecutive days, with the testing arena where two perforated goal boxes were placed in the side chambers as previously described (Dombret et al. 2017). On the day of the test, each male was placed in the neutral chamber and allowed to freely explore each chamber of the testing arena for 10 min. A female treated with DEHP alone or in mixture was placed inside a goal box and randomly assigned to the left or right chamber, while a vehicle-treated female was placed inside a goal box in the other chamber. The number of entries into each compartment and the time spent sniffing each female by the male over the 10-min test were scored.

Y-maze test. Sexually experienced males were allowed to become familiar with the maze for 10 min over 2 consecutive days. On the day of the test, male mice were offered the choice between an anesthetized female from the vehicle group and an anesthetized female from the groups exposed to DEHP alone or in mixture, placed at each end of the maze inside perforated goal boxes.

In the second version of this paradigm, female mice were replaced by their urine collected 1 h before the test. For this purpose, an equivalent volume of urine collected from all females of each treatment group was mixed, and 10 µL of this mix was applied on a piece of filter paper. On the day of the test, male mice were offered the choice between a filter paper containing the urine from the vehicle group and a filter paper containing the urine from the groups exposed to DEHP alone or in mixture, placed at each end of the maze inside perforated goal boxes.

For both tests, the time spent by males in chemo-investigation of each stimulus and the number of entries into each arm were scored during the 10-min test.

Ultrasonic vocalizations. Each male was tested in its home cage in the presence of a female from one of the four treatment groups as previously described (Capela et al. 2018, 2019; Dombret et al. 2017). After the introduction of a female, vocalizations were recorded for 4 min with an UltraSoundGate microphone (Avisoft Bioacoustics), which was connected to an ultrasound recording interface plugged into a computer equipped with the Avisoft-SASLab Pro (version 5.2.09; Avisoft Bioacoustics) recording software. Vocalizations were analyzed using Avisoft-SASLab Pro (Avisoft Bioacoustics). Spectrograms were generated for each detected call (frequency resolution: FFT-length: 512; frame size: 100%; overlap: 50%). The parameters used for the automatic quantification of the vocalizations were: cutoff frequency of 30 kHz, element separation based on an automatic single threshold with a hold time of 15 ms. Syllables were identified and grouped into three main categories (simple, complex, frequency-jump). The total number and duration of USVs were analyzed, as well as the number and duration of each syllable.

Locomotor activity. The computed circular corridor used to measure activity was made of two concentric cylinders crossed by four diametrically opposite infrared beams (Dombret et al. 2017; Raskin et al. 2009). The locomotor activity was counted when animals interrupted two successive beams and had thus traveled a quarter of the circular corridor. Activity was recorder for 120 min and was expressed as cumulative activity over the whole 120-min test.

Body and uterine weight measurements. BW of female mice of the second cohort was monitored weekly during the whole period of treatment and behavioral analyses. At the end of behavioral experiments, animals were sacrificed by pentobarbital injection (120 mg/kg), and the uterus was collected and weighed. The results were expressed as absolute body and uterine weights, and as relative uterine weight (percentage of BW).

Immunohistochemistry

Brains from perfused animals were post-fixed overnight in 4% paraformaldehyde in phosphate buffered saline (PBS). Brains were then sliced into coronal sections of 30 µm in a vibratome and processed for immunolabeling. Sections were blocked for 2 h with 2% normal donkey serum (Sigma-Aldrich) in PBS that contained 0.3% Triton-X100, then incubated with polyclonal anti-ERα antibody diluted at 1:400 (Santa Cruz Biotechnology) or polyclonal anti-PR antibody diluted at 1:400 (Dako-Agilent) for 72 h at 4°C. Immunofluorescence was performed with an Alexa Fluor 488-conjugated chicken antirabbit secondary antibody diluted at 1:500 (Life Technologies-Invitrogen) for 2 h at room temperature in the dark. After several rinses with PBS, sections were rinsed in water, mounted in Mowiol® and stored at 4°C in the dark. Sections were scanned using a high-resolution NanoZoomer Hamamatsu scanner (Hamamatsu Corporation). The number of labeled cells and mean fluorescence per section were counted by NDP.view (NDP.view2, Hamamatsu, Hamamatsu Corporation) and ImageJ software (ImageJ 1.53, NIH; Abràmoff et al. 2004), respectively, in anatomically matched sections identified using the Mouse Brain Atlas (Paxinos and Franklin 2001). ERα- and PR-immunoreactive cells were analyzed in the medial amygdala within an area of 0.56 mm^2 (plate 46), in the bed nucleus of stria terminalis within an area of 0.70 mm^2 (plate 30), in the ventromedial hypothalamus within an area of 0.12 mm^2 (plate 46), in the medial preoptic area within an area of 0.80 mm^2 (plate 30), in the arcuate nucleus within an area of 0.20 mm^2 (plate 46) and in the posteromedial cortical amygdala within an area of 0.56 mm^2 (plate 46).

Statistics

Data were expressed as means ± S.E.M., except for those reporting the percentage of females exhibiting lordosis behavior, and analyzed by GraphPad Prism 6 (GraphPad Software).

Normality tests (Kolmogorov-Smirnov and Shapiro-Wilks tests) were performed. Two-way analysis of variance (ANOVA) was used to analyze the main effect of exposure and stimulus on the number of entries for olfactory preference. The Kruskal-Wallis test was used to analyze the effect of exposure on the estrous cycle and stage durations, BW, lordosis and rejection quotients, the number of syllables (short, upward, one-jump), the duration of syllables (short, upward, downward, modulated, mixed, one-jump, and frequency-jump), the number of ERα-immunoreactive cells in the medial amygdala and arcuate nucleus, and the number of PR-immunoreactive cells in the medial amygdala and bed nucleus of stria terminalis. Dunn's post hoc tests were used to determine group differences. Student's one-sample *t*-test with 0 as the theoretical value was used to analyze the discrimination index for olfactory preference. Partner preference was analyzed by the Student's paired *t*-test or the Wilcoxon test. One-way ANOVA was used to analyze the effect of exposure on the remaining data. Bonferroni's post hoc tests were used to determine group differences. *p*-Values of <0.05 were considered to be significant.

Results

Effects of Adult Female Mice Exposure to DEHP Alone or in Mixture on the Estrous Cycle

Adult female mice were exposed orally for 6 wk, and analyses of the estrous cycle were started while maintaining the treatment for a further 7 wk. Figure 1A shows five consecutive estrous cycles represented for one female from each treatment group. Females exposed to DEHP alone or in phthalate mixture had cycles with longer durations compared with the control female. Quantitative analyses showed an effect of treatment on the mean cycle duration ($p=0.0001$), with significant longer durations for DEHP-5 (+33%; $p=0.0003$), DEHP-50 (+35%; $p=0.0001$) and Mix groups (+20%; $p=0.027$) than control females (Figure 1B). Detailed analyses of the duration of each stage showed different effects depending on the stage of the estrous cycle. An effect of treatment was observed on the proestrus stage ($p=0.0001$), with a shorter duration in the DEHP-5 (−12%; $p=0.0021$), DEHP-50 (−11%; $p=0.0016$), and Mix groups (−16%; $p=0.0001$) compared with control females (Figure 1C). There was also an effect of treatment on the estrus ($p=0.0001$) and metestrus durations ($p=0.0001$), whereas the diestrus stage remained unaffected ($p=0.77$). In particular, a longer duration of the estrus stage was noticed for the three treatment groups (+64% for the DEHP-5 group, +38% for the DEHP-50 group, and +52% for the Mix group compared with the control group). Similarly, the metestrus stage was longer for the DEHP-5 (+87%) and DEHP-50 groups (+97%) in comparison with control females.

Monitoring of BW during the whole period of treatment and estrous cycle analyses showed no significant differences between the treatment groups (Figure S2A). After estrous cycle analyses, body and uterine weights were measured at the metestrus stage. There was no effect of treatment on BW ($p=0.48$; Figure 1D), but an effect on uterine weight was detected ($p=0.04$), with a mean increase of 25% in comparison with the control group (Figure 1E). Post hoc analyses did not show significant differences between the treatment groups.

Effects of Adult Mice Exposure to DEHP Alone or in Mixture on Lordosis Behavior and Olfactory Preference

To determine the behavioral effects of DEHP alone or in a phthalate mixture at comparable hormonal levels, all the following analyses were performed on ovariectomized females, which received implants containing similar estradiol levels. Females were administered progesterone 4–5 h before each behavioral test to induce their receptivity.

Lordosis behavior was first analyzed in naive (Test 1) and sexually experienced females (Test 2), in response to mounts of sexually experienced males as presented in Figure 2A. The percentage of females showing at least a lordosis posture was not statistically different between the four treatment groups for Tests 1 and 2, although a tendency toward lower percentages was observed in females exposed to DEHP alone or in phthalate mixture (Figure 2B). The quantification of the lordosis quotient showed no effect of treatment on Test 1 ($p=0.21$), but a significant effect on Test 2 ($p=0.001$) (Figure 2C). Post hoc analyses showed a lower quotient of the DEHP-5, DEHP-50 and Mix groups in females, in comparison with the control group (−55%, $p=0.0045$; −56%, $p=0.0049$; −54%, $p=0.0062$, respectively). This was mainly because behavior in Test 2 was improved in control females (+147% vs. Test 1), whereas it remained low in the three other treated groups. The rejection quotient in Test 2 was also affected by treatment ($p=0.0201$), but not in Test 1 ($p=0.0931$), with a significant higher quotient in the DEHP-50 group (+271%, $p=0.0281$ vs. the control group) (Figure 2D).

Female sexual behavior is activated by olfactory cues emitted by the male partner. We tested the ability of females to discriminate between male and female odors in preference tests using gonadally intact males vs. sexually receptive females (Figure 2E). In this Y-maze paradigm, the total time spent sniffing the stimuli was equivalent for females from the four exposure groups ($p=0.53$) (Figure 2F). Two-way ANOVA of the number of entries into each arm showed no effect of stimulus ($F_{(1,45)}=1.75$, $p=0.19$) or treatment ($F_{(3,45)}=2.06$, $p=0.12$) (Figure 2G). In contrast, there was an effect of treatment on the olfactory discrimination index ($p=0.0289$) (Figure 2H). Females exposed to DEHP-50 or to the mix showed no preference for males over females, whereas a preference was observed for the control and DEHP-5 groups.

Effects of Adult Mice Exposure to DEHP Alone or in Mixture on Female Ability to Attract Male Partners

In the three-chamber test, a sexually experienced male was presented with two awake females, one from the control group and the other from the group treated with DEHP alone or in mixture. Each female was placed in one of two opposite compartments, separated by a neutral one (Figure 3A). The number of entries into each compartment was similar for the three experimental conditions (control vs. DEHP-5, control vs. DEHP-50, and control vs. Mix) (Figure 3B). An analysis of the percentage of time spent investigating each female showed that males spent more time investigating control females than DEHP-5, DEHP-50, or Mix groups ($p=0.0006$, $p=0.0015$, and $p=0.046$, respectively) (Figure 3C).

To understand why females treated with DEHP alone or in phthalate mixture were less attractive than their control littermates, the same females were anesthetized to avoid any social interaction and analyzed in a Y-maze paradigm. Males were again given the choice between two groups of females (Figure 3D). No differences were observed in the number of entries into the stimulus arms (Figure 3E). In contrast, there were differences in the percentage of time spent investigating each female (Figure 3F), with males spending less time sniffing DEHP-5, DEHP-50, and Mix groups than control females ($p=0.005$, $p=0.0011$, and $p=0.019$, respectively).

These results strongly suggested that the differences observed in the investigation by males were probably related to the olfactory cues emitted by exposed females. To confirm this hypothesis, we performed another Y-maze test where the stimuli consisted of urine collected from mice of the four treatment groups (Figure 3G). Data illustrated in Figure 3H–I show comparable numbers of entries

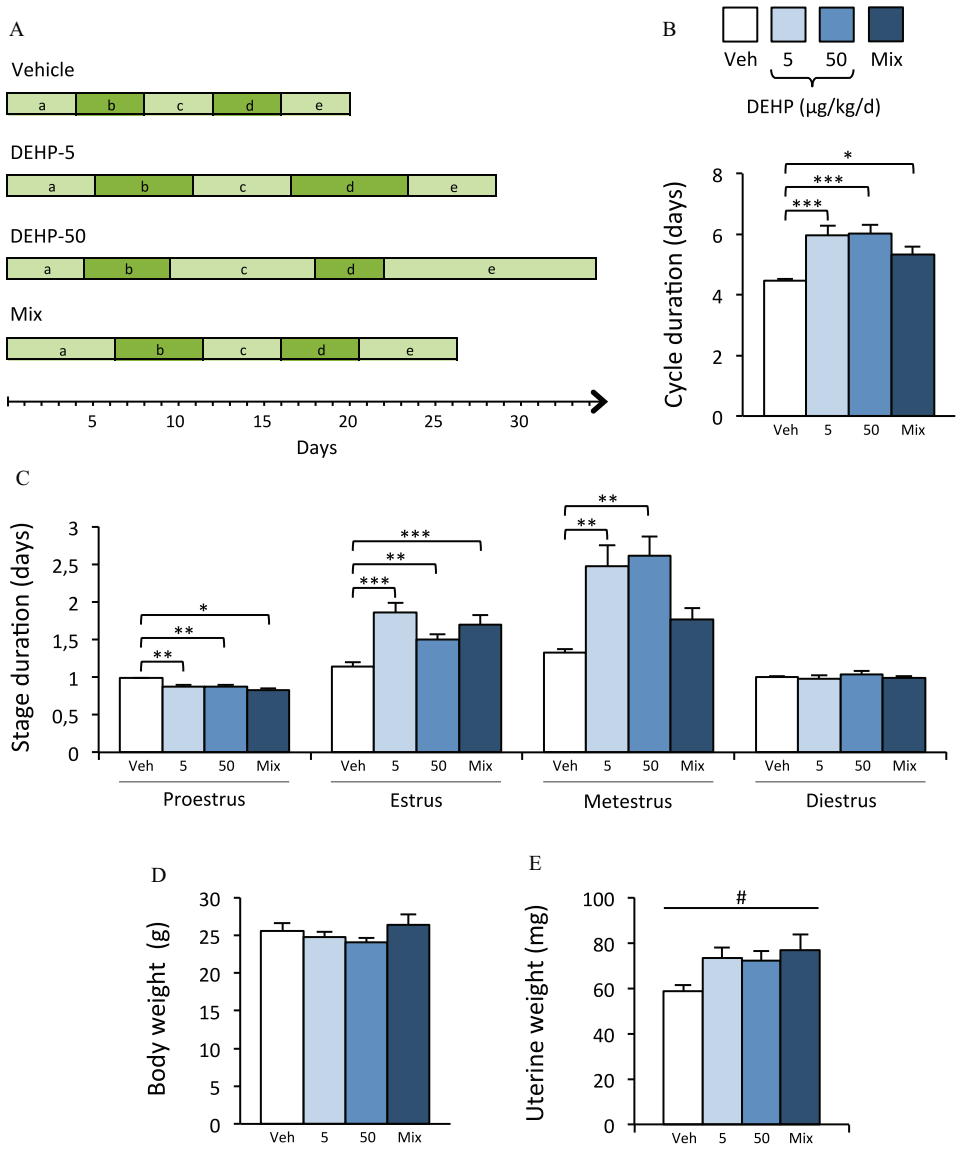

Figure 1. Effects of adult female mice exposure to DEHP alone or in mixture on estrous cyclicity. A. Representation of five consecutive estrous cycles (a–e) in 4 females exposed either to the vehicle (control), DEHP at 5 (DEHP-5) or 50 µg/kg/d (DEHP-50), or to a phthalate mixture (Mix). The duration of the estrous cycles (in days) are indicated. (B–C) Mean duration of the estrous cycle (B) and mean duration of each stage of the estrous cycle (C) in female mice. Data expressed as means ± S.E.M for 15 females per treatment group. Kruskal-Wallis analysis showed a treatment effect on the duration of the estrous cycle ($p = 0.0001$), proestrus, estrus, and metestrus ($p = 0.0001$). Post hoc analyses ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. the control group) are indicated. (D–E). Body (D) and uterine weights (E) are indicated as means ± S.E.M. Treatment effect on uterine weight shown by one-way ANOVA ($^{\#}p < 0.05$). Summary data for panels B, C, D, and E can be found in Table S3. Note: ANOVA, analysis of variance; DEHP, di(2-ethylhexyl) phthalate; SEM, standard error of the mean.

into the arms, but less time spent in investigation for DEHP-5, DEHP-50, and Mix groups in comparison with the control group ($p = 0.0049$, $p = 0.0066$, and $p = 0.015$, respectively).

Effects of Exposure to DEHP Alone or in Mixture on the Emission of Male USVs

We compared the ability of control vs. DEHP- or Mix-exposed females to stimulate the emission of courtship USVs by males. Recordings during the 4-min of interaction between the sexual partners showed an effect of treatment on the total number of syllables ($p = 0.0023$), with a significant lower number in DEHP-50 and Mix groups vs. the control group (−27%, $p = 0.047$; −41%, $p = 0.0013$, respectively) (Figure 4A). Detailed analyses of each syllable shows an effect of treatment on the total number of short ($p = 0.039$), downward ($p = 0.022$), complex ($p = 0.041$), mixed ($p = 0.023$), and frequency-jump ($p = 0.005$) syllables (Figure 4B–D). An effect of treatment was also observed on the total duration of syllables ($p = 0.031$), with a lower duration in the Mix group (−51% vs. the control group, $p = 0.028$) (Figure 4E). Significant treatment effects were also seen on the total duration of downward ($p = 0.028$), complex ($p = 0.022$), and mixed ($p = 0.034$) syllables (Figure 4F–H).

Effects of Female Mice Exposure to DEHP Alone or in Mixture on General Behavior and Parameters

Locomotor activity was measured in a circular corridor (Figure S3). An effect of treatment ($p = 0.016$) was observed on cumulative

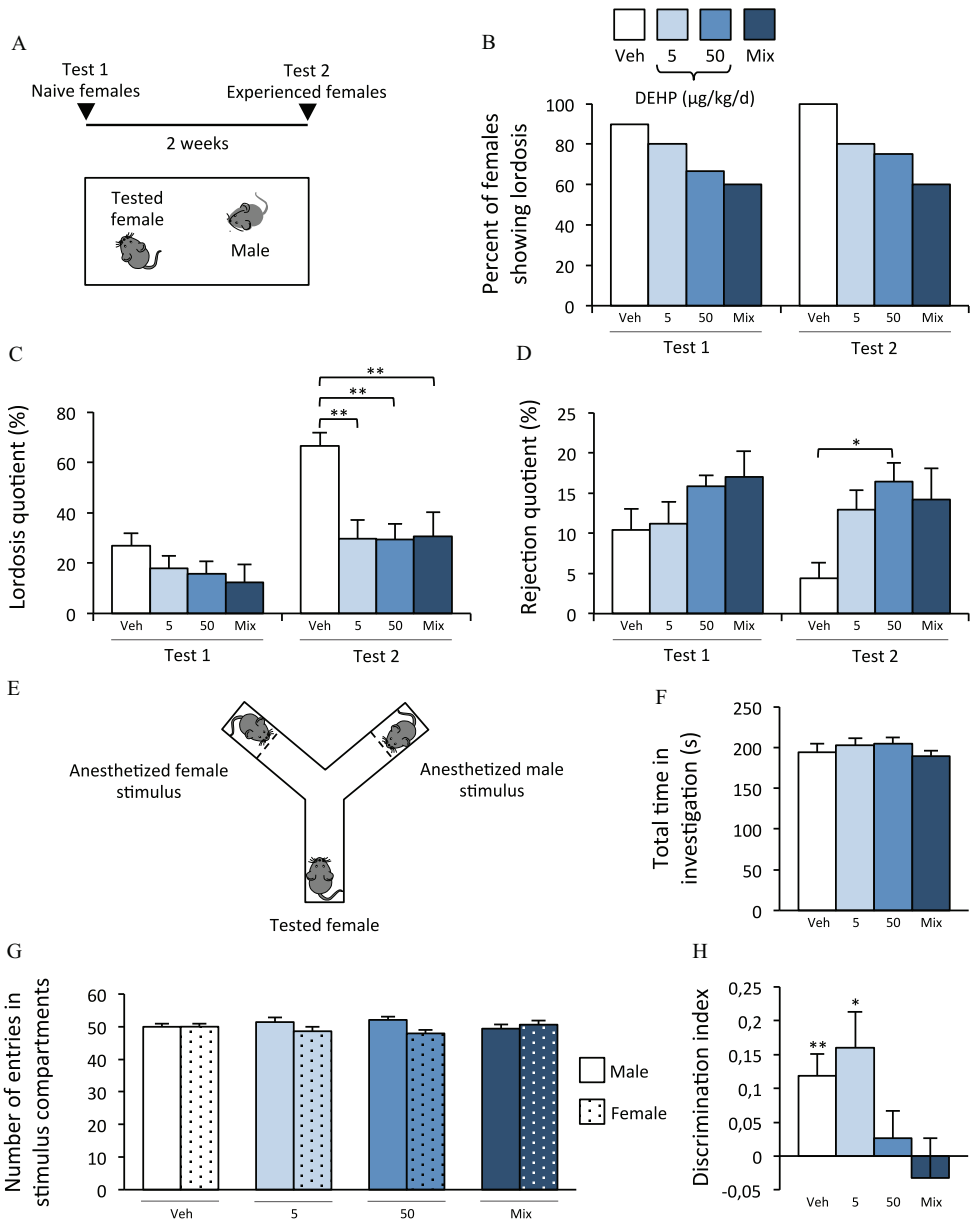

Figure 2. Effects of adult female mice exposure to DEHP alone or in mixture on lordosis behavior and olfactory preference. (A) Lordosis behavior was tested in naive (Test 1) and experienced (Test 2) females in the presence of a sexually experienced male with a two-week interval duration. (B) Percentage of female mice ($n = 11$–13 per treatment group) showing lordosis behavior in the four treatment groups exposed to the vehicle (Veh, control), DEHP at 5 or 50 µg/kg/d or to a phthalate mixture (Mix). (C) Lordosis quotient, number of female lordosis posture/number of male mounts, was calculated in Tests 1 and 2 for the four treatment groups (means ± S.E.M). Kruskal-Wallis analysis showed a treatment effect of treatment for Test 2 ($p = 0.001$); post hoc analyses ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. the control group) are indicated. (D) Rejection quotient, number of female rejection behavior/number of male mounts, was calculated in Tests 1 and 2 for the four treatment groups. Kruskal-Wallis analysis showed a treatment effect for Test 2 ($p = 0.0201$); post hoc analyses ($^{*}p < 0.05$ vs. the control group) are indicated. (E–H). Olfactory preference of females toward an anesthetized male and female was measured in a Y-maze paradigm (E). Total time spent in chemo-investigation by female mice (F) and number of entries into the male and female stimulus arms (G) are presented as means ± S.E.M. The discrimination index (H), time spent by exposed females in male investigation minus the time spent in female investigation divided by the total time of investigation, is expressed as means ± S.E.M. One-way ANOVA showed a treatment effect on the discrimination index ($p = 0.0289$); positive index for the control and DEHP-5 groups ($^{*}p < 0.05$ and $^{**}p < 0.01$) are indicated. Summary data for panels B, C, D, F, G and H can be found in Table S4. Note: ANOVA, analysis of variance; DEHP, di(2-ethylhexyl) phthalate; SEM, standard error of the mean.

activity during the 120-min test. Post hoc analyses showed a lower activity (−32%) vs. the control group for the Mix group ($p = 0.0097$).

The BW of females was monitored during the whole period of treatment and behavioral analyses (Figure S2B); no significant effects of treatment were observed. Furthermore, the absolute and relative weights of uteri, which are known estrogen-dependent organs, were not different among the treatment groups (Table S1), confirming the comparable hormonal levels administered to ovariectomized female mice.

Effects of Exposure to DEHP Alone or in Mixture on the Neural Circuitry That Underlies Female Sexual Behavior

Females exposed to DEHP alone or in an environmental mixture exhibited several impaired components of sexual behavior, under normalized estrogen and progesterone levels. We asked whether these behavioral alterations were due to a direct effect of phthalates on the neural signaling pathways for these hormones. For this, we analyzed ERα- and PR-immunoreactivity in the neural structures underlying this behavior.

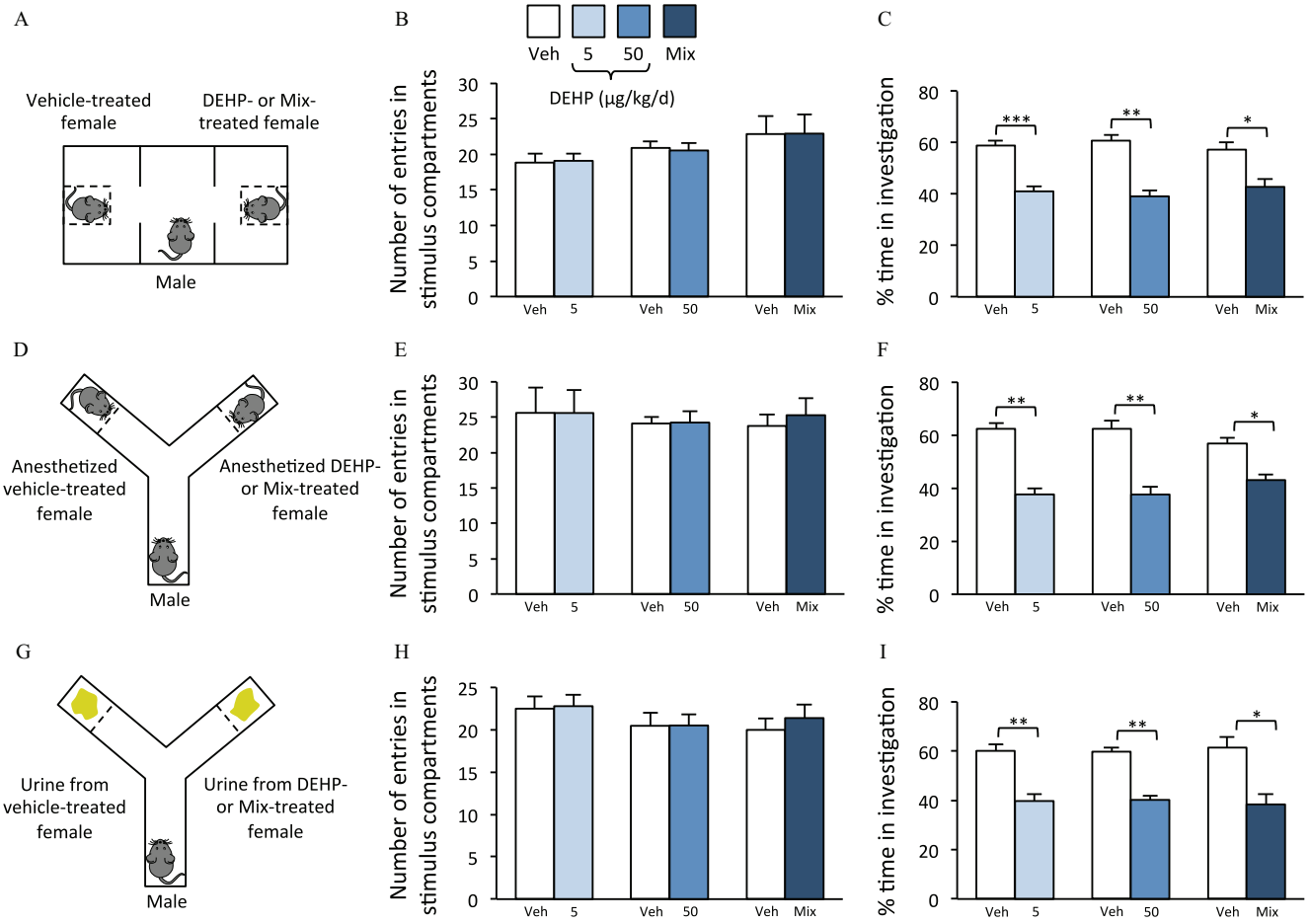

Figure 3. Effects of adult female mice exposure to DEHP alone or in mixture on partner preference. (A–C) In a three-chamber test, a sexually experienced male had choice between a control female exposed to the Veh and a female exposed to DEHP alone or in mixture (A). The number of entries of males into the chamber of control female vs. the chamber of female exposed to DEHP at 5 (DEHP-5) or 50 µg/kg/d (DEHP-50), or to a phthalate mixture (Mix) (B), and the percentage of time spent investigating each female (C) are represented as means ± S.E.M. Males were used for 11–13 females per treatment group. Paired *t*-test or Wilcoxon test are indicated ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. the control group). (D–F) In a Y-maze test, a sexually experienced male had the choice between an anesthetized female exposed to the Veh and a female exposed to DEHP alone or in mixture (D). The number of entries of males into the arm of Veh vs. the arm of DEHP- or Mix-exposed female (E) and the percentage of time spent investigating each female (F) are presented as means ± S.E.M. Paired *t*-test or Wilcoxon test ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. the control group). (G–I) In a Y-maze paradigm, a sexually experienced male had the choice between urine from vehicle-exposed females and urine from females exposed to DEHP alone or in mixture (G). The number of entries of males into the arm of Veh vs. the arm of DEHP or Mix-exposed urine (H) and the percentage of time spent investigating the Veh vs. DEHP or Mix-exposed female urine (I) are presented as means ± S.E.M. Paired *t*-test or Wilcoxon test ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. the control group) are indicated. Summary data for panels B, C, E, F, H, and I can be found in Table S5. Note: DEHP, di(2-ethylhexyl) phthalate; SEM, standard error of the mean; Veh, vehicle.

A specific nuclear ERα signal was detected in neurons of the medial and posteromedial cortical amygdala, bed nucleus of stria terminalis and ventromedial hypothalamus corresponding to the principal facilitatory system (Figure 5A–C; Figure S4A, left). Quantitative analyses showed no significant effect of treatment on the number of ERα-immunoreactive neurons (Figure 5D–F; Figure S4B, left), or mean fluorescence density in these brain areas (Figure 5G–I, Figure S4B, right).

A specific nuclear PR-immunoreactivity was also observed in all these cerebral structures except the posteromedial cortical amygdala where the signal was hardly detected (Figure 6A–C; Figure S4A,-right). Quantitative analyses showed a significant effect of treatment on the number of PR-immunoreactive neurons in the medial amygdala ($p < 0.0001$), bed nucleus of stria terminalis ($p = 0.0096$), and ventromedial hypothalamus ($p = 0.0010$) (Figure 6D–F). Post hoc analyses showed a lower number of PR-immunoreactive neurons for the three treated groups in the medial amygdala (−27% to −44%), the bed nucleus of stria terminalis (−44% to −55%), and the ventromedial hypothalamus (−25% to −38%). Similar results were obtained for the mean fluorescence density (Figure 6G–I).

Comparable immunohistochemical analyses of the inhibitory system of lordosis behavior, including the medial preoptic area and arcuate nucleus, were carried out. The data obtained showed no effect of treatment on the number of ERα-immunoreactive neurons or mean fluorescence density in the medial preoptic and arcuate nuclei (Figures 7A,C; Figure S4C). In contrast, effects of treatment were observed on the number of PR-immunoreactive neurons in the medial preoptic area ($p = 0.0003$) and arcuate nucleus ($p = 0.0428$) (Figures 7B,D), with a significant lower number in all treatment groups (−28% to −42% in the medial preoptic area, −20% to −41% in the arcuate nucleus, in comparison with the control group). Similar results were observed for the mean fluorescence density (Figure S4D).

Discussion

The present study shows for the first time to our knowledge that chronic exposure of adult female mice to low doses of DEHP alone

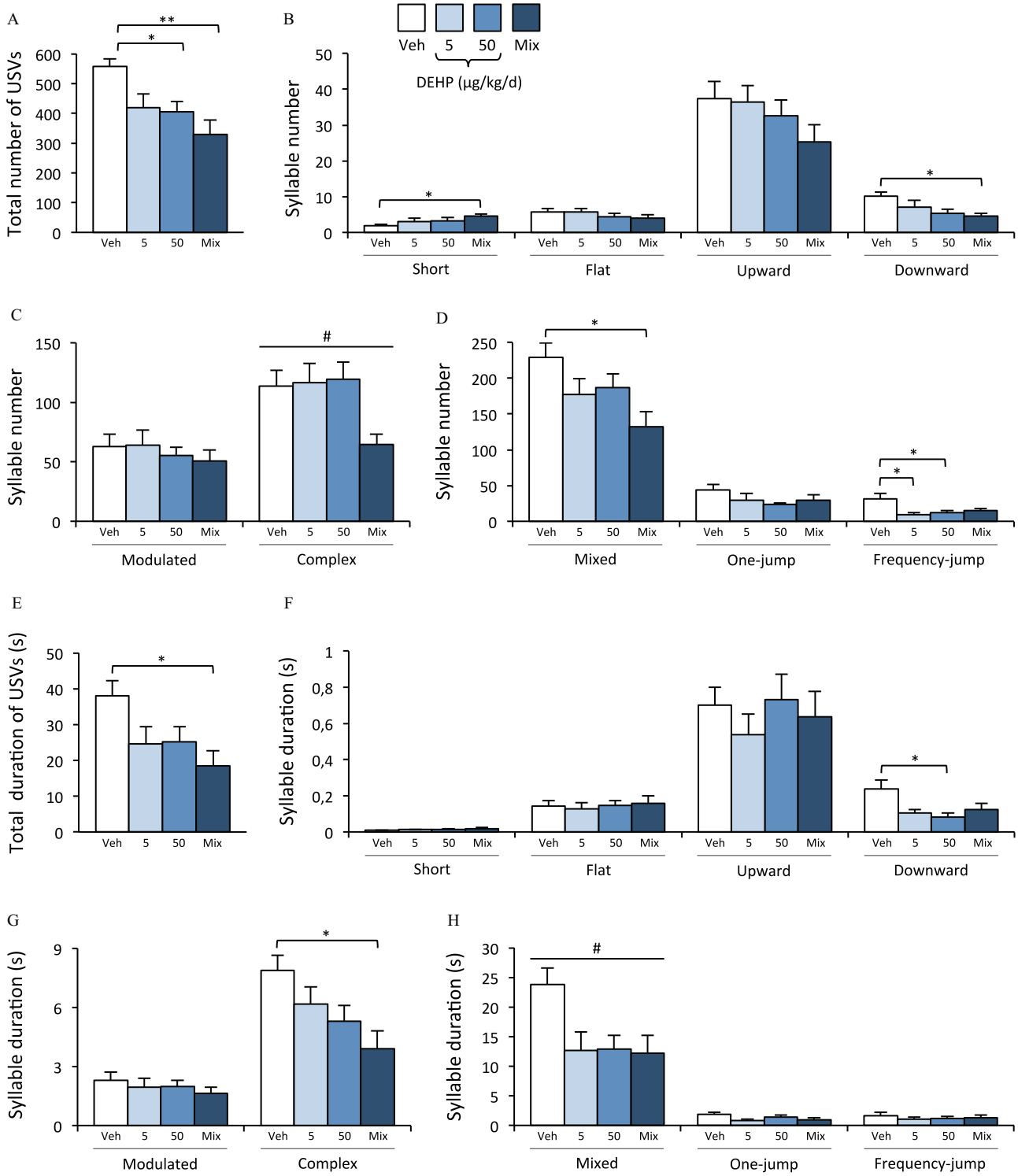

Figure 4. Effects of adult female mice exposure to DEHP alone or in mixture on the emission of male USVs. (A) Total number of USV emitted by sexually experienced males in the presence of females exposed to the Veh, DEHP at 5 or 50 µg/kg/d, or to a phthalate mixture (Mix) during the 4-min recording. Data are expressed as means ± S.E.M. Males were used for 11–13 females per treatment group. (B–D) Total number (means ± S.E.M) of syllables of the simple (B), complex (C), and frequency jump (D) category. A significant treatment effect was found by one-way ANOVA on the number of total USVs ($p = 0.0023$), downward ($p = 0.022$), complex ($p = 0.041$), mixed ($p = 0.023$), and frequency-jump syllables ($p = 0.005$), and by Kruskal-Wallis on the number of short syllables ($p = 0.039$). Post hoc analyses ($^{*}p < 0.05$, $^{**}p < 0.01$ vs. the control group) are indicated. (E) Total duration (means ± S.E.M) of ultrasonic vocalizations emitted by males. (F–H) Total duration (means ± S.E.M) of syllables of the simple (F), complex (G), and frequency jump (H) categories. A significant treatment effect was found by one-way ANOVA on the duration of total USVs ($p = 0.031$) and complex syllables ($p = 0.022$), by Kruskal-Wallis for the duration of downward ($p = 0.028$) and mixed syllables ($\sharp p = 0.034$); post hoc analyses ($^{*}p < 0.05$ vs. the control group) are indicated. Summary data for panels A, B, C, D, E, F, G, and H can be found in Table S6. Note: ANOVA, analysis of variance; DEHP, di(2-ethylhexyl) phthalate; SEM, standard error of the mean; USV, ultrasonic vocalization; Veh, vehicle.

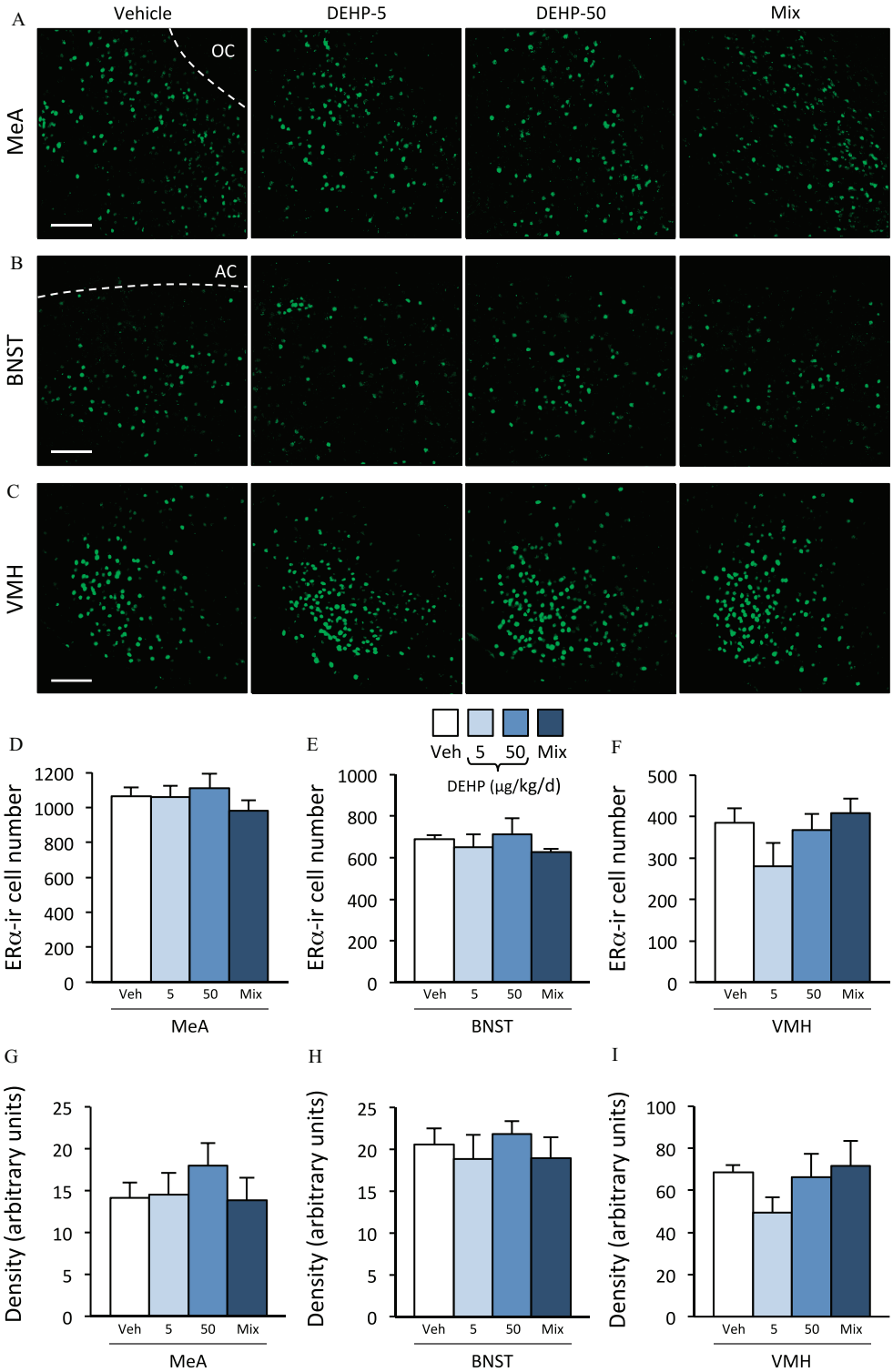

Figure 5. Effects of adult exposure to DEHP alone or in mixture on neural ERα-immunoreactivity in the facilitatory system of female mice. (A–C) Representative nuclear ERα-immunolabeling in the MeA (A), BNST (B), and VMH (C) of females exposed to the Veh, DEHP at 5 (DEHP-5) or 50 μg/kg/d (DEHP-50), or to a phthalate mixture (Mix). Scale bar: 100 μm; (D–I). Quantitative analyses of the number of ERα-immunoreactive (ir) cells (D–F) and mean fluorescence density (G–I) in the indicated brain areas. Data are means ± S.E.M. of 6 females per treatment group. Summary data for panels D, E, F, G, H, and I can be found in Table S7. Note: AC, anterior commissure; BNST, bed nucleus of stria terminalis; DEHP, di(2-ethylhexyl) phthalate; MeA, medial amygdala; OC, optic chiasma; PR, progesterone receptor; SEM, standard error of the mean; Veh, vehicle control; VMH, ventromedial hypothalamus.

or in an environmental phthalate mixture impaired reproductive behavior. Exposed females exhibited a lowered ability to discriminate between male and female pheromones and ability to attract males and induce the emission of male courtship vocalizations. They also displayed a lower lordosis quotient and inversely a higher rejection behavior, in response to male mounts. These behavioral alterations were associated with a significantly lower number of PR-immunoreactive neurons in the neural structures

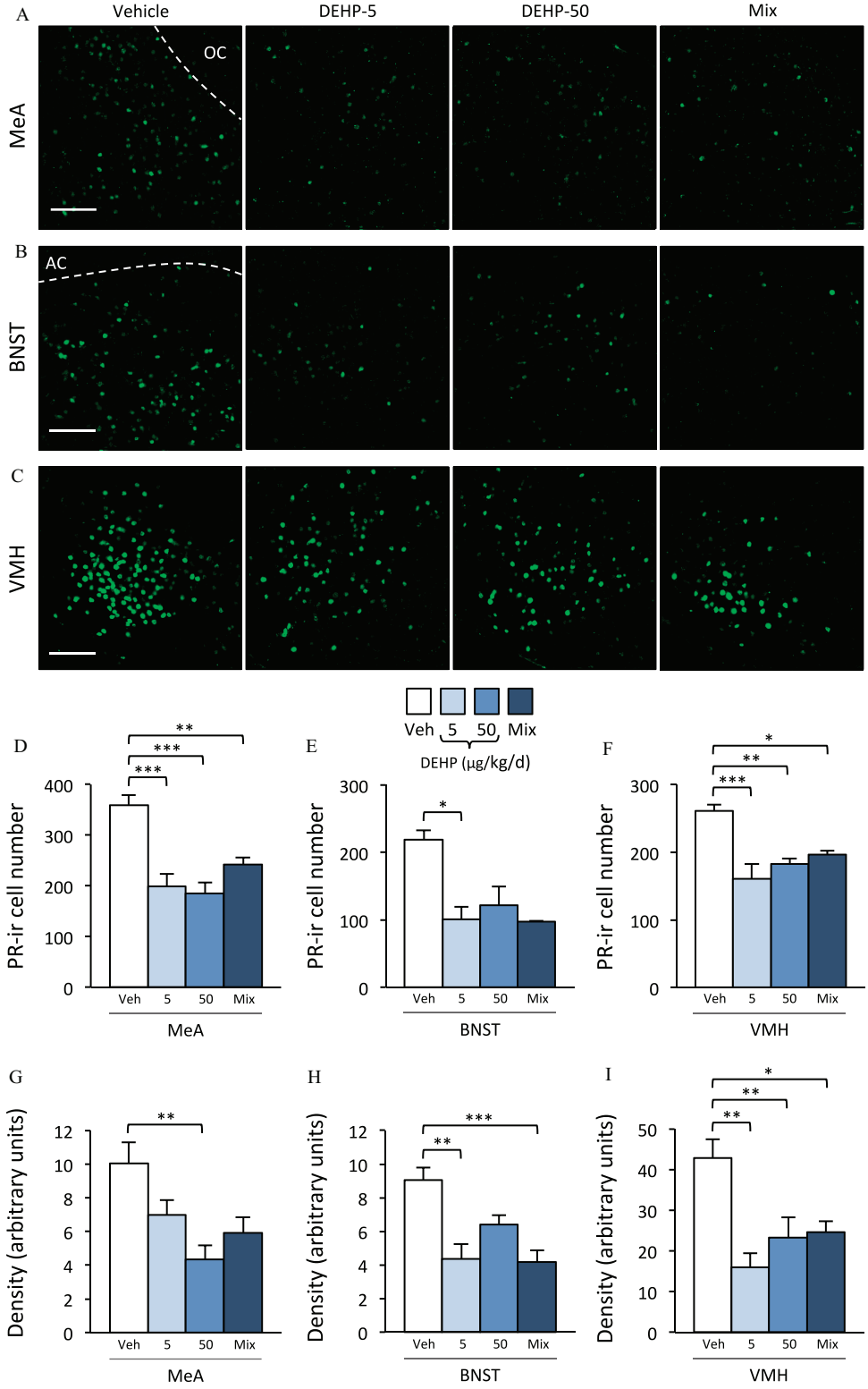

Figure 6. Effects of adult female mice exposure to DEHP alone or in mixture on neural PR-immunoreactivity in the facilitatory system. (A–C) Representative nuclear PR-immunolabeling in the MeA (A), BNST (B), and VMH (C) of females exposed to the Veh, DEHP at 5 (DEHP-5) or 50 µg/kg/d (DEHP-50), or to a phthalate mixture (Mix). Scale bar: 100 µm; (D–I) Quantitative analyses of the number of PR-immunoreactive (ir) cells (D–F) and mean fluorescence density (G–I) in the indicated brain areas. Data are means ± S.E.M. of 6 females per treatment group. A significant treatment effect was found by one-way ANOVA on cell number in the MeA ($p < 0.0001$) and VMH ($p = 0.0010$), and on density in the BNST ($p = 0.0003$) and VMH ($p = 0.0022$), whereas Kruskal-Wallis analysis showed a treatment effect on cell number in the BNST ($p = 0.0096$) and density in the MeA ($p = 0.0107$). Post hoc analyses ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. the control group) are indicated. Summary data for panels D, E, F, G, H, and I can be found in Table S8. Note: AC, anterior commissure; ANOVA, analysis of variance; BNST, bed nucleus of stria terminalis; DEHP, di(2-ethylhexyl) phthalate; MeA, medial amygdala; OC, optic chiasma; PR, progesterone receptor; SEM, standard error of the mean; Veh, vehicle control; VMH, ventromedial hypothalamus.

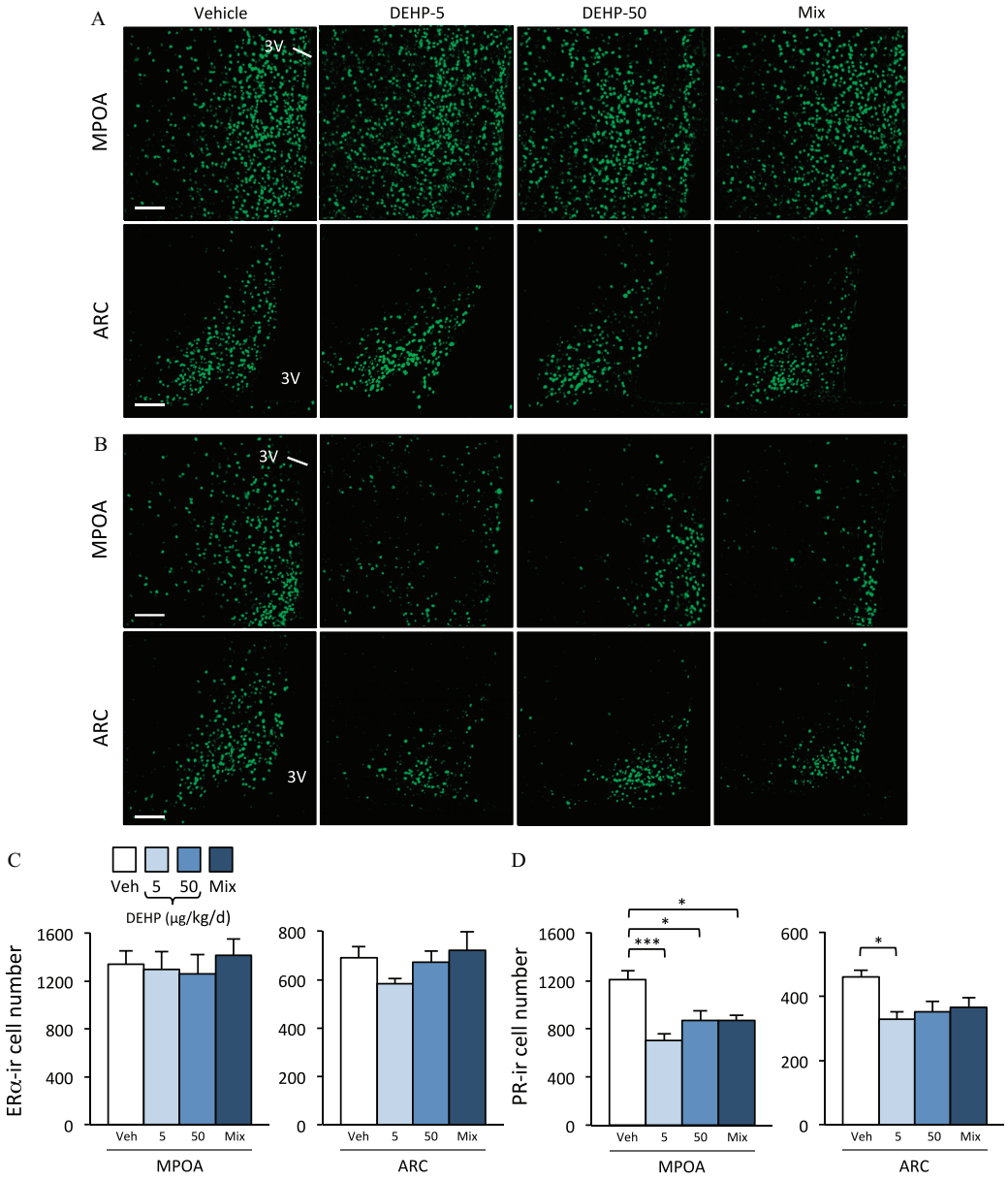

Figure 7. Effects of adult female mice exposure to DEHP alone or in mixture on neural ERα- and PR-immunoreactivity in the inhibitory system. (A–B) Representative nuclear ERα- (A) and PR-immunolabeling (B) in the MPOA and ARC of female mice exposed to the Veh, DEHP at 5 (DEHP-5) or 50 µg/kg/d (DEHP-50), or to a phthalate mixture (Mix). Scale bar: 100 µm; 3V: third ventricle. (C–D) Quantitative analyses of the number of ERα- (C) and PR-immunoreactive (ir) cells (D) in the indicated brain areas. Data are means ± S.E.M. of 6 females per treatment group. A significant treatment effect was found by one-way ANOVA on the number of PR-immunoreactive cells in the MPOA ($p = 0.0003$) and ARC ($p = 0.0428$). Post hoc analyses ($^{*}p < 0.05$, $^{***}p < 0.001$ vs. the control group) are indicated. Summary data for panels C and D can be found in Table S9. Note: ANOVA, analysis of variance; ARC, arcuate nucleus; DEHP, di(2-ethylhexyl) phthalate; ER, estrogen receptor; MPOA, medial preoptic area; PR, progesterone receptor; SEM, standard error of the mean; Veh, vehicle control.

underlying female sexual behavior. In the majority of analyses, the effects observed were similar between DEHP at the TDI dose (50 µg/kg/d) and at a 10-fold lower dose of 5 µg/kg/d, and between DEHP alone at 5 µg/kg/d and in the phthalate mixture.

In response to male mounts, female mice exposed to DEHP alone or in mixture exhibited a low lordosis quotient and a higher rejection behavior after a first sexual experience. In comparison, sexually experienced females of the control group showed a higher lordosis and a lower rejection quotient. The analysis of general behavior showed an effect of treatment on locomotor activity monitored in the circular corridor, with a significantly lower activity in the Mix group. Although lower activity during mating can participate in the lowered sexual behavior of females from the Mix group in particular, it is not sufficient to explain the altered mating behavior of the other treatment groups. The altered lordosis behavior can be at least partly due to the lower olfactory preference because exposed females were unable to discriminate between male and female pheromonal cues, in particular for the DEHP-50 and Mix groups. It is, however, interesting to note that DEHP-5 group females also had a low lordosis quotient despite unchanged olfactory discrimination. Altogether, these data suggest that exposure to DEHP alone or in phthalate mixture impaired plasticity of the neural areas involved in female sexual behavior processing. The observed alterations were probably located at both the level of the olfactory system and chemosensory areas located downstream from the olfactory system and involved in behavioral processing.

Females exposed to DEHP alone or in mixture were also less efficient than females of the control group in attracting males. In the three-chamber test using awake nonanesthetized animals, males preferred to investigate females of the control group. In these experimental conditions where behavior was recorded during the dark phase, sexual partners interacted through the emission of pheromones by the two partners and USVs mainly by males. When they are receptive, females emit specific pheromones that stimulate male behavior such as chemo-investigation and courtship USVs (Bean 1982; Dizinno and Whitney 1977; Nyby et al. 1977; for review: Mhaouty-Kodja 2020). An interesting finding is that a similar lower chemo-investigation by males was observed when anesthetized females, or their urine, were presented to chemo-investigation by males. In addition, in the presence of females exposed to DEHP alone or in phthalate mixture, males emitted a lower number of USVs for a shorter duration, with a significant impact on 5 of the 9 categories of syllables (short, downward, complex, mixed, and frequency-jump).

Altogether, these data indicate that exposure to DEHP alone or in phthalate mixture probably altered the pheromonal cues emitted by females, which then impaired attraction of the male partner and stimulation of USV emission.

Olfactory cues from female and male mice including bodily and urinary excretions play an essential role during mating. As mentioned above, female olfactory cues inform the male mouse about the receptive status of the female and induce sexual arousal of the male partner. Although some sociosexual pheromones such as the exocrine-gland-secreting peptide 1 (Kimoto et al. 2007) or major urinary proteins (Kaur et al. 2014) have been identified in males, less is known about female pheromones that are necessary during mating. Sulfated estrogens derived from metabolization of estrogens during the estrus phase were characterized as potential signal molecules in the female urine (Nodari et al. 2008). These sulfated estrogens emitted by ovulating females were shown to activate male vomeronasal receptors and promote male courtship behavior (Haga-Yamanaka et al. 2014; Nodari et al. 2008). In this context, it is possible that exposure to DEHP alone or in phthalate mixture altered peripheral estradiol metabolism, thereby resulting in modified excretion of sulfated estrogens in the urine of receptive females. In support of this hypothesis, a recent study described that exposure to a low-dose mixture of food contaminants containing DEHP altered the expression of estrogen sulfotransferase, the enzyme that catalyzes the formation of sulfated estrogens (Naville et al. 2013).

To determine the molecular mechanisms underlying the effects of exposure to DEHP alone or in phthalate mixture on female behavior, we analyzed the whole neural circuitry involved in the activation of female sexual behavior. The behavioral alterations induced by exposure to these molecules could not be due to differences in the levels of estradiol and progesterone, the key hormones underlying behavioral activation. Indeed, levels of these hormones were normalized in all females tested through ovariectomy and hormonal supplementation. We thus quantified the number of neurons expressing ERα and PR, the two key receptors mediating estradiol- and progesterone-induced regulation of sexual behavior. An interesting finding was that a selective effect of treatment on the number of PR-expressing neurons was observed, whereas the number of ERα-immunoreactive neurons remained unaffected. A significantly lower number of PR-expressing neurons was observed in both the facilitatory (medial amygdala, bed nucleus of stria terminalis, ventromedial hypothalamus) and inhibitory (medial preoptic area, arcuate nucleus) systems of lordosis behavior in females exposed to DEHP alone or in mixture. These differences can fully explain modified female behavior because ERα-mediated up-regulation of PR is required for the induction of female receptivity in rodents (Parsons et al. 1981; for review: Mhaouty-Kodja et al. 2018).

Two previous studies reported effects of phthalates at high doses on PR expression in females. Perinatal exposure of female rats to diisononyl phthalate (20,000 ppm) lowered PR mRNAs levels in the medial preoptic area (Takagi et al. 2005). A significant increase in the nuclear levels of PR was observed in epithelial breast cancer cells exposed to 10 μM DEHP (Crobeddu et al. 2019). Besides its function in female reproduction, the PR has been shown to play a key role in neuroprotection and promyelination (González et al. 2020; Schumacher et al. 2014). It would be interesting to study whether and how adult exposure to environmental doses of phthalates alters PR expression in other female reproductive functions and nonreproductive systems.

The down-regulation of neural PR and lowered behavior in females are comparable with our results obtained recently in males, where adult exposure to DEHP at the same doses influenced behavior and induced androgen receptor (AR) down-regulation in the neural structures underlying male sexual behavior (Table S2), without effect on hormonal levels (Dombret et al. 2017). Such observations indicate that DEHP alone or in an environmental phthalate mixture were able to directly affect the nervous system. Another intriguing observation is that DEHP exposure seems to target a close subfamily of neural steroid receptors, i.e., AR in males and PR in females. Indeed, according to the phylogeny of the steroid receptor gene family, AR and PR are pairs of closely related sister receptors among the second steroid receptor subgroup, which also includes glucocorticoid and mineralocorticoid receptors (Eick and Thornton 2011). The first subgroup is formed by ERα and ERβ (Eick and Thornton 2011). Whether *AR* and *PR* genes share common regulatory mechanisms of expression that could be similarly disrupted by DEHP exposure needs further investigation.

Analyses of intact females showed that adult exposure to DEHP alone or in an environmental phthalate mixture impaired the progression of the estrous cycle. A shorter proestrus phase and longer estrus and metestrus phases were observed in females exposed to phthalates. Effects on the estrous cycle were described for 10 d or 16 wk of adult exposure to high doses of DEHP (0.5 to 2 g/kg/d), with a prolonged estrous cycle and delayed ovulation in rats and mice (Davis et al. 1994; Li et al. 2012). Adult exposure to DEHP at 20 μg/kg/d for 10 d or at 200 mg/kg/d for 30 d also prolonged the duration of the estrus phase (Hannon et al. 2014). In comparison, our data indicate that effects on the estrous cycle can be observed at lower doses of DEHP (5 μg/kg/d) alone or in a phthalate mixture after 6 wk of exposure. This alteration was associated with a higher uterine weight, suggesting hormonal modifications, in agreement with previous studies showing altered ovarian estrogen and progesterone levels and pituitary LH and FSH levels following exposure to DEHP in female mice (Chiang et al. 2020; Li et al. 2012) or sheep (Herreros et al. 2013).

The present results showing effects at different levels of the female reproductive system (estrous cycle, behavior) suggest that the impact of exposure to DEHP alone or in mixture on female reproduction should be important. In comparison, our previous studies in males showed an effect of DEHP exposure on reproductive behavior, but not on the integrity of the HPG axis (Dombret et al. 2017). Given the behavioral effects induced by adult exposure to DEHP in the two sexual partners (Figure 8), major adverse effects on mating in rodents may be expected. These effects may be extended to other vertebrate species where sexual reproduction is tightly regulated by sex steroid receptors. In humans, despite some differences with rodents in the control of the cycle, the key endocrine mechanisms important for reproduction success involve also a crosstalk among the ovary, the hypothalamus,and the pituitary (Viguié et al. 2018). At the

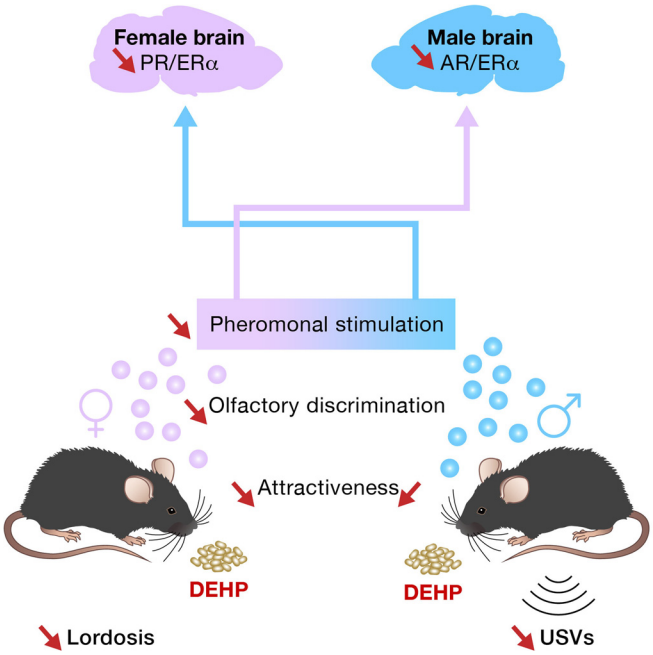

Figure 8. Proposed mechanisms for behavioral effects of adult exposure to DEHP in sexual partners. Chronic oral exposure of male and female mice to DEHP affected the physiological balance between the key neural sex steroid signaling pathways in the neural circuitry underlying sexual behavior. A lower expression of neural AR in males and neural PR in females occurred without effects on neural ERα expression. The resulting lower neural AR/ERα ratio in males might be related to a lower emission of USVs and the ability to attract female partners. In females, the lower neural PR/ERα ratio was associated with a lower olfactory discrimination and expression of lordosis behavior. In addition, modifications in female pheromonal cues probably due to peripheral effects of phthalates were related to a lower ability of exposed females to stimulate male courtship behavior such as USV emission. This suggests that combined modifications induced by DEHP exposure in both sexual partners may greatly impact mating and sexual reproduction in rodents. The red arrows represent the lowered sex steroid receptor ratio and male and female behaviors induced by adult DEHP exposure. Note: AR, androgen receptor; DEHP, di(2-ethylhexyl) phthalate; ER, estrogen receptor; PR, progesterone receptor; USVs, ultrasonic vocalizations.

behavioral level, sexual function in women is under this hormonal control (Mills et al. 2019). Altogether, these observations suggest that the effects induced in mice by adult exposure to low doses of phthalates on cyclicity and behavior might be relevant for human reproduction. It is interesting to note that epidemiological studies reported associations between exposure to phthalates and reproductive issues, including premature ovarian failure (Hlisníková et al. 2020) and low interest in sexual activity (Barrett et al. 2014). In this context, it is important to indicate that since 1975 sexual health has been included by the World Health Organization (WHO) as part of reproductive health, and it is recognized that sexual dysfunction may affect not only reproduction but also mental and general health, well-being, and maturation (WHO 2006).

The neuroendocrine and behavioral effects induced by DEHP alone or in phthalate mixture were observed following continuous exposure of adult females. In males, our recent work shows that exposure arrest for 2 months completely reversed the modifications triggered by DEHP exposure in behavior and hypothalamic AR expression (Capela and Mhaouty-Kodja 2021). Whether female neural structures also present this high plasticity after arrest of exposure will be assessed in further studies.

DEHP effects were observed at low doses of 5 or 50 μg/kg/d. Except for rejection behavior of experienced females, olfactory discrimination and total USV number for which a more efficient effect was triggered by the DEHP dose of 50 μg/kg/d for, the other behaviors and parameters were similarly affected by the two doses. The lack of dose response may be because the two doses differ only by a 10-fold factor; additional lower and higher doses are maybe needed to observe such dose–response. This hypothesis is supported by our previous data obtained on males and showing similar effects induced by the doses of 5 and 50 μg/kg/d for partner preference, mating, and neural androgen receptor protein amount whereas the dose of 0.5 μg/kg/d was inefficient (Dombret et al. 2017). The results also suggest that DEHP at 5 μg/kg/d drives the effects in the phthalate mixture. This was probably due to the fact that DEHP was the predominant phthalate in the mixture with a concentration 10- to 20-fold higher than the other phthalates.

The majority of experimental studies addressing exposure to phthalates including DEHP have used high doses of these molecules. The TDI dose of 50 μg/kg/d for DEHP was established by the EFSA 2005 on the basis of the ability of this molecule to reduce fetal testosterone production, whereas the oral reference dose of 20 μg/kg/d established by the U.S. Environmental Protection Agency in 1987 was determined for an increased liver weight (U.S. EPA 1987). A recent revision by the EFSA of five phthalates including DEHP maintains the TDI dose at 50 μg/kg/d according to effects on fetal testosterone production (EFSA 2019). Our studies at the neural level addressing adult exposure (Dombret et al. 2017; and in the present study) and some studies concerning recent evidence reporting *in vivo* peripheral effects at doses equivalent or below the reference doses for prenatal (Abdel-Maksoud et al. 2019; Barakat et al. 2019) or adult exposure (Hannon et al. 2014; Lu et al. 2019) reveal the high sensitivity of both female and male reproductive systems. These data should lead to the consideration of several end points of male and female reproduction and maybe later to the revision of reference values established for a single end point.

Conclusion

This study shows for the first time to our knowledge that chronic exposure of adult female mice to DEHP alone or in an environmental phthalate mixture interfered with several components of sexual behavior. Exposed females displayed lower lordosis behavior and olfactory preference. They also showed a lower ability to attract and stimulate male behavior may be due to modifications of emitted pheromonal cues by exposed females. Together with the altered duration of estrous cyclicity, this finding led us to suggest that under comparable experimental conditions (doses used, exposure period and duration), reproduction of females was more vulnerable than that of their male congeners to adult exposure to phthalates. The behavioral alterations induced and driven by DEHP in the phthalate mixture were probably caused at least in part by PR down-regulation in the neural structures underlying female sexual behavior. All the behavioral and neural effects observed were induced by doses of DEHP equivalent to or below the TDI dose. This indicates that the nervous system is highly sensitive to these compounds and should be considered in this context as a relevant end point in risk assessment for these molecules.

Acknowledgments

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